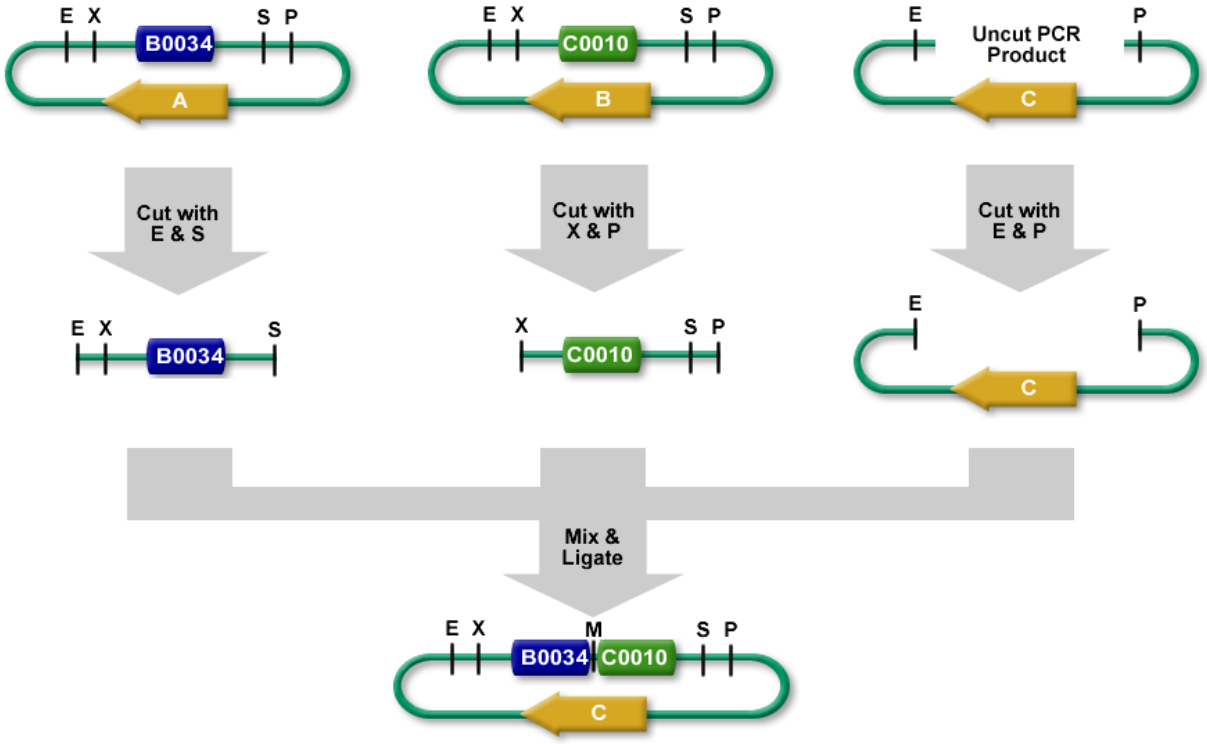
PROTOCOL FOR CLONING

(Modified 3A Assembly iGEM Protocol)

The main requirement of this protocol is to have different antibiotic resistance genes in the primary (cut out plasmids) and the end products (cloned plasmid), depicted in the figure below which is taken from iGEM website.



MATERIALS:

- Miniprepped DNA from two samples (Sample A and Sample B)
- iGEM provided Linearized plasmid backbone (with a different resistance to the plasmid backbones of Sample A and Sample B)
- EcoRI, XbaI, SpeI and PstI
- T4 DNA ligase buffer
- T4 DNA ligase
- CutSmart Buffer
- MilliQ Water

PROCEDURE:

1. **Digestion**-

Enzyme master mix for linearized plasmid backbone (25 µL for 5 reactions):

CutSmart Buffer	5 µL
EcorI	0.5 µL
PstI	0.5 µL
MilliQ Water	19 µL

Digest Backbone: Add 4 µL of this master mix into 4 µL of iGEM provided linearized plasmid backbone (25 ng/µL for 100 ng in total) in PCR tubes and mix well with pipette.

Enzyme master mix for DNA from Sample A (25 µL for 5 reactions):

CutSmart Buffer	5 µL
EcorI	0.5 µL
SpeI	0.5 µL
MilliQ Water	19 µL

Digest DNA from Sample A: Add 4 µL of this master mix into 4 µL of miniprepped DNA from Sample A (25 ng/µL for 100 ng in total) in PCR tubes and mix well with pipette. Adjust with MilliQ water if miniprepped concentration is higher than the 25 ng/µL.

Enzyme master mix for DNA from Sample B (25 µL for 5 reactions):

CutSmart Buffer	5 µL
XbaI	0.5 µL
PstI	0.5 µL
MilliQ Water	19 µL

Digest DNA from Sample B: Add 4 µL of this master mix into 4 µL of miniprepped DNA from Sample B (25 ng/µL for 100 ng in total) in PCR tubes and mix well with pipette. Adjust with MilliQ water if miniprepped concentration is higher than the 25 ng/µL.

Digest all three reactions (in three separate tubes) at 37 ^{o}C for 30 minutes and then heat kill at 80 ^{o}C for 20 minutes either manually or in the thermocycler.

Take 2-3 µL of these digested products for ligation and use rest of the products for Digestion analysis with the help of gel electrophoresis in order to confirm successful digestion of the DNA.

2. **Ligation**-
   Add 2 µL (25 ng) of digested backbone in a separate PCR tube.
   Add equimolar amount of digested samples from A and B (should be <3 µL) to it.
   Mix well and then add 1 µL of T4 DNA ligase buffer.
   Add 0.5 µL of T4 DNA ligase.
   Mix well and add MilliQ water upto 10 µL.

   Ligate at 16 ^{o}C for 30 minutes and then heat kill at 80 ^{o}C for 20 minutes either manually or in the thermocycler.

   Take 2 µL of this ligated product for transformation.

3. **Transformation**-
   See "Transformation Protocol"

4. **Colony PCR**-
   Colony PCR is done to find out bacterial colonies with the right construction plasmid.
   See "Colony PCR protocol"

5. **Overnight cultures and Restriction analysis**-
   As colony PCR was inconclusive in most of the cases in our lab, we tried to analyze cloned plasmids through an alternative method i.e. Overnight cultures (from several transformed colonies) followed by Restriction analysis. This is a time consuming method.
   See "Overnight cultures and Restriction analysis".